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Research Note

Differentiation between Mixed Infections of *Ancylostoma caninum* and *Ancylostoma duodenale* in Dogs Using an In Vitro Assay for the Resumption of Feeding by Third-stage Infective Larvae

J. M. Hawdon and G. A. Schad
Department of Pathobiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

ABSTRACT: The identification of 2 mixed hookworm infections in dogs by using differences of larval in vitro feeding behavior is reported. In the first case, infective third-stage larvae (L_3) from a putative *Ancylostoma duodenale* infection were compared to larvae of known *A. duodenale* and *A. caninum* infections in a resumption of feeding time course experiment. The second case involved comparison of feeding behavior of L_3's from a second suspect infection with L_3's from a known *A. caninum* infection at 24 hr. In both cases, the suspect larvae exhibited feeding behavior similar to known *A. caninum* larvae, suggesting accidental infection with *A. caninum*. Upon necropsy, the suspect infections contained adults of both species. This technique has potential application in differentiation of closely related nematode species in several areas of parasitology.

KEY WORDS: *Ancylostoma caninum, Ancylostoma duodenale,* infective larvae, in vitro feeding.

As long ago as 1924, epidemiologists had hoped to distinguish among the infective larvae of the dog hookworm, *Ancylostoma caninum* (Ercolani, 1859), and the human hookworms, *Ancylostoma duodenale* (Dubini, 1843) and *Necator americanus* (Stiles, 1903). They concluded that although *N. americanus* is distinguishable from the others on morphological grounds, the 2 *Ancylostoma* species are not (Schuurmans Stekhoven and Schuurmans Stekhoven-Meyer, 1924; Komiya and Yasuraoka, 1966). In our laboratory, both species of *Ancylostoma* have been maintained in dogs for more than 15 yr (Schad, 1979), and on rare occasions donor animals have inadvertently been infected with larvae of the wrong species, or with a mixed inoculum. Until recently, dogs harboring putatively pure infections, but with atypical prepatent periods or egg counts, had to be sacrificed, and the adult hookworms recovered and examined morphologically to determine the true nature of the infection. This is a very expensive procedure due to the high cost of purpose-bred dogs. A reliable method of distinguishing between the larvae of the 2 hookworms would obviate the unnecessary termination of an infection. We have found that

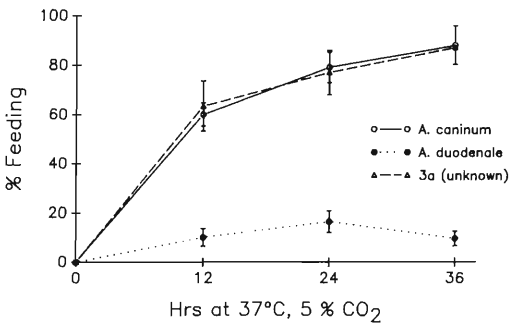

Figure 1. Differentiation of hookworm species using an in vitro assay of L_3 feeding. Larvae are assayed for feeding by the method of Hawdon and Schad (1990). Larvae cultured from the feces of donor dog 3a were compared to larvae cultured from known *A. caninum* and *A. duodenale* donors. Each point is the mean ± standard deviation of 3 replicates.

Table 1. Resumption of feeding by third-stage larvae of a known *Ancylostoma caninum* infection, and infective larvae from donor F151, a potentially mixed infection of *A. caninum* and *A. duodenale*.*

Strain	10% canine serum	Mean % feeding ± SD†
A. caninum	+	73.8 ± 8.0[a]
	−	11.4 ± 2.4[b]
F151	+	74.6 ± 9.5[a]
	−	1.4 ± 1.3[b]

* L_3's incubated in 0.1 ml RPMI ± 10% normal canine serum at 37°C, 5% CO_2 for 24 hr. After incubation, feeding larvae are labelled as described elsewhere (Hawdon and Schad, 1990).

† Mean of 3 replicates ± standard deviation (SD). Values with different superscripts are significant at $P < 0.01$ using the Student's *t*-test. Statistics are done on arcsin transformed data, and the retransformed means expressed as percentages.

third-stage larvae (L_3) of the 2 species behave differently in an in vitro feeding assay (Hawdon and Schad, 1990), and have employed this assay to determine the species identity of suspect hookworm infections on 2 occasions.

In the first infection, donor beagle 3a was inoculated with approximately 4,000 L_3, believed to be *A. duodenale*. Fecal egg counts were not performed between days 13 and 18 postinfection, by which time the infection was patent. Although generally 25–30 days, a prepatent period of 18 days was close to the range reported for *A. duodenale* infections (Leiby et al., 1987). However, the initial egg output of 5,200 eggs per gram (epg), and a peak egg output on day 11 postpatency of 29,600, were extraordinarily high for a pure *A. duodenale* infection in our canine model system, casting further doubt as to the purity of the infection. Consequently, the suspect donor's feces were collected and cultured separately from those of a known *A. duodenale* infected dog. After 10 days incubation, L_3's were harvested from known pure cultures of *A. caninum* and *A. duodenale* and from cultures of the suspect infection. Larvae were assayed for feeding at 12, 24, and 36 hr incubation as described elsewhere (Hawdon and Schad, 1990). The results are shown in Figure 1. The *A. caninum* larvae showed characteristic species-specific feeding behavior, with a maximum proportion of the larvae having resumed feeding by 24 hr incubation. In sharp contrast, few larvae from the pure *A. duodenale* larvae initiated feeding by 24 hr. Larvae from the suspect donor 3a exhibited feeding behavior identical to that of the known *A. caninum* larvae, suggesting accidental contamination of the infection with this species. At necropsy, 110 adult hookworms were recovered, and 22 of the smallest worms (i.e., in the size range of *A. duodenale* in the dog) were identified by counting the number of ventral teeth in the buccal cavity. Nineteen of the examined worms were *A. caninum,* and 3 were *A. duodenale,* confirming the suspected mixed nature of the infection.

A second helminth-naive beagle (donor F151) was inoculated with approximately 1,000 putative *A. duodenale* larvae. The prepatent period was 18–22 days, and the initial egg output was 4,600 epg at day 22 postinfection, reaching a peak of 17,400 epg on day 37. These parameters suggested contamination of the infection with *A. caninum.* Again, feces from the suspect donor were cultured separately, and L_3's were recovered after 10 days. These larvae, together with 10-day known *A. caninum* larvae, were assayed for feeding after 24 hr incubation. At the time of comparison there were insufficient *A. duodenale* larvae to assay. The results suggested that the suspect larvae were *A. caninum,* since the known *A. caninum* larvae were feeding at 73.8 ± 8.0%, and the unknown larvae at 74.6 ± 9.5% (Table 1). Examination of 215 of a total of 248 adult hookworms recovered from the small intestine at necropsy yielded 198 *A. caninum* and 17 *A. duodenale* adults.

These results indicate that it is possible to distinguish between pure and contaminated infections involving 2 closely related species of hookworm in experimentally infected dogs using in

vitro feeding as an assay. Since, to our knowledge, *A. duodenale* is maintained in dogs at only 2 locations worldwide (the other being the Institute of Parasitic Diseases, Hangzhou, Peoples' Republic of China), the need for such a technique in a laboratory situation is probably uncommon. However, this technique may prove useful in epidemiological and epizootological studies. For example, it may be possible to identify mixed infections of the sympatric human hookworms *A. duodenale* and *A. ceylanicum* (Looss, 1911), whose larvae are morphologically very similar (Yoshida, 1971), based on in vitro differences in feeding behavior. In the cases reported here, where 1 species was more successful in establishing in the host than the other, feeding behavior resembled that of the dominant species. However, when co-occurring parasites are similarly vigorous, the feeding behavior of larval populations derived from a mixed infection should be intermediate. Secondly, this technique may be useful in field situations where soil ecologists or epidemiologists have no practical method to distinguish among the common congeneric ancylostomes (*A. duodenale, A. caninum, A. tubaeforme* Zeder, 1800, and *A. braziliense* de Faria, 1910) of humans and their usual companion animals. In industrialized countries, this is particularly important in parks, playgrounds, and other public open spaces, where irresponsible pet ownership with attendant fecal pollution raises the possibility of invasion by nematode larvae associated with cutaneous and visceral larva migrans in humans. Finally, adaptation of this technique to other parasites may aid in the nondestructive differentiation of closely related species inhabiting the same host.

These experiments also suggest that the 2 *Ancylostoma* species may resume feeding in response to different host signals during infection, or more likely, that the larvae respond to a species-specific host stimulus. Although this strain of *A. duodenale* is adapted to the dog (Leiby et al., 1987), larvae may require a signal specific to human serum in order for a large proportion of them to resume feeding in vitro. Comparative investigations of the resumption of feeding in larvae of various hookworm species are currently underway in this laboratory.

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